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Analysis of 16S–23S intergenic spacer regions of the rRNA operons in *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates from fish

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ABSTRACT

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Aims: To analyse interspecies and intraspecies differences based on the 16S–23S rRNA intergenic spacer region (ISR) sequences of the fish pathogens *Edwardsiella ictaluri* and *Edwardsiella tarda*.

Methods and Results: The 16S-23S rRNA spacer regions of 19 *Edw. ictaluri* and four *Edw. tarda* isolates from four geographical regions were amplified by PCR with primers complementary to conserved sequences within the flanking 16S–23S rRNA coding sequences. Two products were generated from all isolates, without interspecies or intraspecific size polymorphisms. Sequence analysis of the amplified fragments revealed a smaller ISR of 350 bp, which contained a gene for $tRNA^{Glu}$, and a larger ISR of 441 bp, which contained genes for $tRNA^{Ile}$ and $tRNA^{Ala}$. The sequences of the smaller ISR of different *Edw. ictaluri* isolates were essentially identical to each other. Partial sequences of larger ISR from several *Edw. ictaluri* isolates also revealed no differences from the one complete *Edw. ictaluri* large ISR sequence obtained. The sequences of the smaller ISR of *Edw. tarda* were 97% identical to the *Edw. ictaluri* smaller ISR and the larger ISR were 96–98% identical to the *Edw. ictaluri* larger ISR sequence. The *Edw. tarda* isolates displayed limited ISR sequence heterogeneity, with ≥97% sequence identity among isolates for both small and large ISR.

Conclusions: There is a high degree of size and sequence similarity of 16S–23S ISR both among isolates within *Edw. ictaluri* and *Edw. tarda* species and between the two species.

Significance and Impact of the Study: Our results confirm a close genetic relationship between *Edw. ictaluri* and *Edw. tarda* and the relative homogeneity of *Edw. ictaluri* isolates compared with *Edw. tarda* isolates. Because no differences were found in ISR sequences among *Edw. ictaluri* isolates, sequence analysis of the ISR will not be useful to distinguish isolates of *Edw. ictaluri*. However, we identified restriction sites that differ between ISR sequences of *Edw. ictaluri* and *Edw. tarda*, which will be useful in distinguishing the two species.

Keywords: bacteria, channel catfish, *Edwardsiella ictaluri*, *Edwardsiella tarda*, intergenic spacer region, restriction fragment length polymorphism.

INTRODUCTION

The genus *Edwardsiella*, belonging to the family Enterobacteriaceae, is presently comprised of three species, *Edwardsiella ictaluri*, *Edwardsiella tarda* and *Edwardsiella hoshinae* (Farmer 2003). *Edwardsiella ictaluri*, primarily a fish pathogen, is the cause of enteric septicaemia of catfish (*Ictalurus punctatus* Rafinesque), a leading cause of mortality in catfish worldwide (Plumb 1999; Wagner *et al.* 2002). Although a few nonictalurid food fish species (tilapia, *Sarotherodon aureus*; Chinook salmon, *Oncorhynchus*

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tahawstscha; and rainbow trout, *O. mykiss*) are susceptible to experimental infection (Plumb and Sanchez 1983; Baxa *et al.* 1990), among food fish species, *Edw. ictaluri* has been isolated from and associated with naturally-occurring disease only from ictalurid species. Infrequently, *Edw. ictaluri* has been isolated from diseased ornamental fish (rosy barbs, *Puntius conchonius*; green knife fish, *Eigemannia virescens;* danio, *Danio devario*) (Kent and Lyons 1982; Waltman *et al.* 1985; Humphrey *et al.* 1986). *Edwardsiella tarda*, predominantly an enteric pathogen of both marine and fresh water fish, has a broader host range. Besides fish, *Edw. tarda* has been associated with enteric diseases and various pathological conditions in humans (Marsh and Gorach 1982; Wilson *et al.* 1989) and several terrestrial animal species (Janda *et al.* 1991). *Edwardsiella hoshinae* has been isolated from birds, reptiles and water (Grimont *et al.* 1980) and sporadically from the faeces of humans. However, its pathogenic significance remains unknown (Janda and Abbott 1993). The three species of *Edwardsiella* are distinguishable based on their biochemical characteristics (Farmer 2003).

Because *Edwardsiella* are prevalent in aquaculture practices throughout the world and have been isolated from a variety of fish species as well as humans and other vertebrate animals, it is of interest to determine whether *Edwardsiella* isolates possess phenotypic or genotypic characteristics that differ among epidemiologically unrelated isolates. Based on the results of multiple studies, *Edw. ictaluri* is considered a single antigenic group (Rogers 1981; Plumb and Klesius 1988; Plumb and Vinitnantharat 1989; Bertolini *et al.* 1990). Comparison of outer-membrane protein profiles of *Edw. ictaluri* isolates from several geographical locations also indicated considerable phenotypic homogeneity (Plumb and Klesius 1988; Newton *et al.* 1990; Klesius and Horst 1991). However, more recent studies have provided evidence that some *Edw. ictaluri* isolates differ antigenically (Lobb *et al.* 1993; Klesius and Shoemaker 1999). Furthermore, differences between *Edw. ictaluri* isolates have been found based on red blood cell agglutination inhibition by the monosaccharide D-mannose (Wong *et al.* 1989; Ainsworth 1993). Additionally, amplified fragment length polymorphism (AFLP) analysis has demonstrated genetic differences between *Edw. ictaluri* isolated from different fish species (Klesius *et al.* 2003).

In contrast to the single serotype found for *Edw. ictaluri*, typing schemes based on somatic (O) and flageller (H) antigens of *Edw. tarda* have identified a diversity of serovar groups within this species (Sakazaki 1984; Tamura *et al.* 1988). Comparison of the extracellular protein profiles by proteomic analysis has identified differences between virulent and avirulent strains (Tan *et al.* 2002). Random amplified polymorphic DNA profiles of *Edw. tarda* isolated from fish and humans from different countries have also demonstrated differences (Nucci *et al.* 2002).

Ribotyping techniques have enabled detection of genetic variations among epidemiologically unrelated isolates of the same species of eubacteria (Kostman *et al.* 1995; Berridge *et al.* 1998; Houpikian and Raoult 2001). The intergenic spacer regions (ISR) between 16S and 23S rRNA genes are considered to be under less evolutionary selective pressure than the 16S and 23S rRNA coding genes and therefore prone to greater genetic variation. The polymorphisms consist mostly of insertions and/or deletions within the ISR (Anton *et al.* 1998; Luz *et al.* 1998) and sequence analysis of ISR has been extremely useful in detecting interstrain (Gutler and Barrie 1995) and interspecies variations (Bourque *et al.* 1995). In this study, we have analysed the sequence diversity in the 16S–23S rRNA ISR of *Edw. ictaluri* and *Edw. tarda* isolated from outbreaks of fish diseases in different geographic locations.

MATERIALS AND METHODS

Bacteria and DNA isolation

The *Edw. ictaluri* and *Edw. tarda* isolates used in this study are listed in Table 1. The identity of each isolate was determined or confirmed by conventional biochemical tests using the identification card (ID-GNI) for Gram-negative bacilli with the VITEK 32 system (bioMerieux Vitek, Hazelwood, MO, USA) and the API 20E test strips (bioMerieux) following the manufacturer's instructions. The probability matrix for identification of Gram-negative aerobic fermentative bacteria (Holmes *et al.* 1986) and the biochemical reaction profiles for identification of enteric groups in the family Enterobacteriaceae (Farmer 2003) were used to interpret the results. All isolates of *Edw. tarda* and *Edw. ictaluri* fermented glucose, maltose, and mannose and caused deamination of phenylalanine. *Edwardsiella ictaluri* and *Edw. tarda* were differentiated by the ability of *Edw. tarda* to produce indole and hydrogen sulfide and to reduce methyl red. Additional phenotypic characterization of these isolates is the subject of another report (V.S. Panangala, C.A. Shoemaker, S.T. McNulty, C.R. Arias and P.H. Klesius, submitted).

Glycerol stocks (20% glycerol in tryptic soy broth) of the organisms maintained at −80°C were initially cultured on blood agar plates (tryptic soy agar with 5% defibrinated sheep blood). Single colonies were picked after incubation for 36 h, transferred to 10 ml of brain–heart infusion medium and cultured at 28°C in a shaker water bath. From logarithmic phase broth cultures, 1·5 ml of culture containing *c.* 2×10^9 bacteria was centrifuged at 5000 *g* for 10 min in a Microfuge 18 centrifuge (Beckman Coulter, Palo Alto, CA, USA). Chromosomal DNA from *Edw. ictaluri* and *Edw. tarda* was isolated using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) and the protocol provided for isolation of genomic DNA from Gram-negative bacteria.

Table 1 *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates used in this study

Species	Isolate	Source	Origin	GenBank accession no.	
				Smaller ISR	Larger ISR
Edw. ictaluri	IA-30-NJ#1	Tadpole madtom*	New Jersey†	AY706725	
Edw. ictaluri	AL-93-75	Catfish	Alabama‡	AY706726	
Edw. ictaluri	016-S99-1911	Catfish	Mississippi§	AY706727	
Edw. ictaluri	017-S99-1914	Catfish	Mississippi	AY706728	
Edw. ictaluri	013-S99-1908	Catfish	Mississippi	AY706729	
Edw. ictaluri	001-S99-1643	Catfish	Mississippi	AY706730	
Edw. ictaluri	003-S99-1760	Catfish	Mississippi	AY706731	
Edw. ictaluri	ALG-03-189	Catfish	Alabama¶	AY706732	
Edw. ictaluri	AL-95-73	Catfish	Alabama	AY706743	
Edw. ictaluri	RE-33**	Vaccine strain	EILO	AY706733	
Edw. ictaluri	ALG-03-58	Catfish	Alabama	AY706734	
Edw. ictaluri	AL-93-58	Catfish	Alabama	AY706735	AY706745
Edw. ictaluri	EILO	Walking catfish††	Thailand‡‡	AY706736	
Edw. ictaluri	ALG-03-190	Catfish	Alabama	AY706737	
Edw. ictaluri	ATCC-33202§§	Catfish	Georgia¶¶	AY706738	
Edw. ictaluri	ALG-99-407	Catfish	Alabama	AY706739	
Edw. ictaluri	S-94-1051	Catfish	Mississippi	AY706740	
Edw. ictaluri	ALG-03-192	Catfish	Alabama	AY706741	
Edw. ictaluri	ALG-03-161	Catfish	Alabama	AY706742	
Edw. tarda	AL-98-87	Catfish	Alabama	AY706722	AY706746
Edw. tarda	AL-97-052	Catfish	Alabama	AY706723	AY706747
Edw. tarda	AU-98-024	Catfish	Alabama	AY706724	AY706748
Edw. tarda	AL-03-032	Bluegill***	Alabama	AY706744	

ISR, intergenic spacer region.
**Noturus gyrinus*.
†Klesius *et al.* (2003).
‡All isolates with the prefix AL/AU were isolated from diseased fish in AL by the Auburn University Fish Diagnostic Laboratory, Auburn University, AL 36849, USA.
§Isolated from diseased catfish in Mississippi. Dr David Wise, Fish Diagnostic Laboratory, Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS 38776, USA.
¶All isolates with the prefix ALG were isolated from diseased catfish in AL. Mr William Hemstreet, Alabama Fish Farming Center, Greensboro, AL 36744, USA.
**Avirulent vaccine strain used in commercial vaccine, derived from EILO; Klesius and Shoemaker (1999).
††*Clarias batracus*.
‡‡Kasornchandra *et al.* (1987).
§§American Type Culture Collection, Manassas, VA, USA; type strain.
¶¶Obtained from ATCC. However, original isolation was from diseased catfish in GA; Hawke *et al.* (1981).
***Lepomis macrochirus*.

PCR amplification

Oligonucleotide primers (5′-TTGTACACCGCCCGTCA-3′ and 5′-GGTACCTTAGATGTTTCAGTTC-3′) (Kostman *et al.* 1995) complementary to conserved regions of the 16S and 23S rRNA coding sequences flanking the ISR were synthesized at the Iowa State University DNA Sequencing and Synthesis Facility, Ames, IA, USA. The PCR mixture (50-μl total volume) contained 1 μl (50 pmol) of each primer, 18 μl of diethyl pyrocarbonate treated water, 25 μl 2X *Taq* PCR Master Mix (Qiagen), and 5 μl containing 0·2–0·3 μg *Edw. ictaluri* or *Edw. tarda* DNA template. A negative control without template was included with each set of PCR reactions. Amplifications were performed in a GeneAmp

2400 thermal cycler (Perkin Elmer, Norwalk, CT, USA) programmed for initial denaturation at 94°C for 5 min, 35 cycles as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. For some reactions, the extension time was increased to 1 or 2 min, and for some reactions with increased extension times, the annealing time was reduced to 5 s. The PCR amplified products (20 μl) were electrophoresed on 3% agarose gels (3 : 1 Nu-Sieve GTG; FMC Bioproducts, Rockport, ME, USA) containing ethidium bromide. A 100-bp DNA ladder (Fisher Scientific, Pittsburgh, PA, USA) was used as molecular size standards. Following electrophoresis for *c.* 2 h at 80 mA, the DNA bands were visualized by UV illumination.

DNA sequencing and analysis

The PCR products corresponding to the 700 and 800-bp size standards were excised and the DNA was eluted from the gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The *c.* 800-bp less intense bands and the *c.* 700-bp more intense bands were eluted separately and submitted for DNA sequencing to the Auburn University Genomics and Sequencing Laboratory, using the same primers used for PCR. Sequences were compared with other sequences in the GenBank using BLASTn (Altschul *et al.* 1997) (http://www.ncbi.nlm.nih.gov/) and to each other using the ContigExpress and AlignX programs of the VectorNTI suite (Informax Invitrogen Life Science Software, Frederick, MD, USA). Restriction enzyme recognition sites were identified and sizes of restriction fragments predicted using Gene Construction Kit software (Textco Biosoftware, Inc., West Lebanon, NH, USA).

Phylogenetic analysis

The 16S rRNA sequences from a wide range of Enterobacteriaceae (Table 3) were aligned using ClustalX. After exclusion of regions of the alignment containing gaps, the most appropriate evolutionary model for maximum likelihood phylogenetic analysis was identified using the Modeltest 3·06PPC programme. The ISR sequences between the tRNA coding sequences and the 23S rRNA coding sequences from a wide range of Enterobacteriaceae (Table 3) were aligned with those of *Edw. ictaluri*. Three regions of sequence conservation were identified (see Fig. 4). These three regions were concatenated and aligned using ClustalX. The most appropriate evolutionary model for maximum likelihood phylogenetic analysis was identified using the Modeltest 3·06PPC programme. Maximum likelihood and nearest neighbour joining analyses were performed using PAUP* 4·0b10 for Macintosh (Swofford 2002) and the settings identified by Modeltest.

RESULTS

ISR PCR products

The ISR PCR products of all *Edw. ictaluri* and *Edw. tarda* isolates tested yielded indistinguishable patterns upon electrophoresis. Each isolate generated a major *c.* 700-bp PCR product and a less-abundant *c.* 800-bp PCR product (Fig. 1 and not shown). Sequence analysis revealed that the *c.* 700-bp products included ISR of 350 bp, which contained a $tRNA^{Glu}$ gene (Fig. 2), while the *c.* 800-bp products included ISR of 441 bp, which contained $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (Fig. 3).

Comparison of small ISR sequences

The sequences of the smaller ISR from different *Edw. ictaluri* isolates were essentially identical to each other. The only differences among isolates were in the degree of heterogeneity at individual positions in the sequences. It is likely that the PCR products of the same size from each isolate were derived from more than one ribosomal operon and that the heterogeneity in each sequence is a result of variability among those operons within an isolate. Five of the *Edw. ictaluri* isolates (IA-30-NJ#1, RE-33, AL-93-58, EILO and ATCC-33202) each exhibited marked heterogeneity in the number of Ts (three or four) at positions 230–233 and number of As (two or three) at positions 291–293 in the alignment shown in Fig. 2. The other 14 *Edw. ictaluri* isolates had predominately four Ts and three As at these positions, while the *Edw. tarda* isolates, with the exception of isolate AL-98-87, had three Ts and two As at these positions. The heterogeneity in some of the *Edw.*

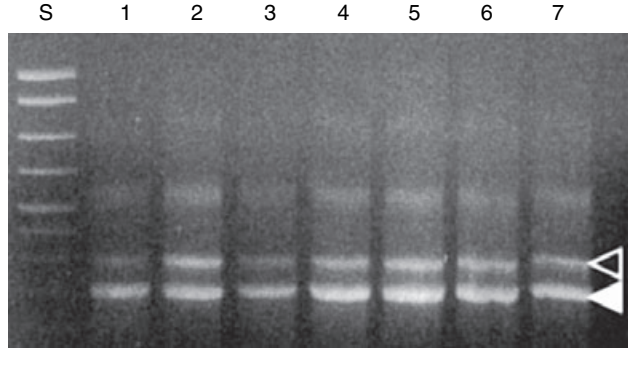

Fig. 1 The PCR amplification products of 16S–23S rRNA ISR of seven *Edwardsiella ictaluri* isolates. Lane S, 100-bp ladder size standard; lane 1, IA-30-NJ1; lane 2, AL-93-75; lane 3, 016-S99-1911; lane 4, 017-S99-1914; lane 5, 013-S99-1908; lane 6, 011-S99-1643; lane 7, 003-S99-1760. White arrowhead indicates the major *c.* 700-bp products; open arrowhead indicates the less-abundant *c.* 800-bp products. All other *Edw. ictaluri* and *Edwardsiella tarda* isolates shown in Table 1 yielded the same band pattern

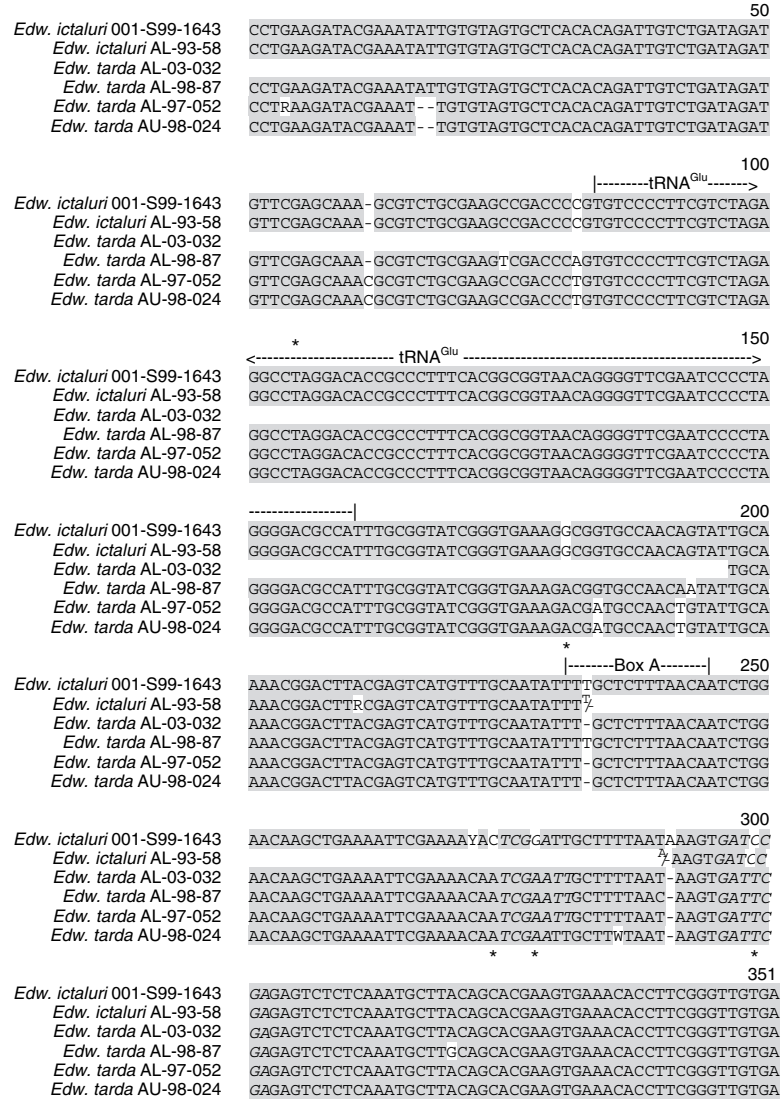

Fig. 2 Aligned sequences of *Edwardsiella ictaluri* and *Edwardsiella tarda* smaller ISR. Dashes indicate gaps introduced to improve alignment. Blanks indicate sequence not determined. Shading indicates identical nucleotides in a majority of the sequences. Asterisks below the aligned sequences indicate positions where all *Edw. tarda* sequences determined were identical to each other but differed from those of *Edw. ictaluri*. Italics indicate restriction enzyme recognition sites present only in either *Edw. ictaluri* or *Edw. tarda* sequences, potentially useful for distinguishing between *Edw. ictaluri* and *Edw. tarda* (see Table 2). Some of the recognition sites are overlapping. Two representative *Edw. ictaluri* sequences are shown. Sequences of the smaller ISR of the *Edw. ictaluri* isolates listed in Table 1 fell into two groups. The first group (including 14 isolates) had sequences identical to the 001-S99-1643 sequence shown (the sequence of one of these isolates, AL-95-73, was determined only for positions 209–328 and was identical to 001-S99-1643 in those positions). The second group (including IA-30-NJ1, RE-33, AL-93-58, EILO and ATCC-33202 isolates) had sequences identical to AL-93-58 for the positions shown. Each of the sequences in the second group had heterogeneity in the number of Ts and As at positions 230–233 and 291–293 respectively. This is indicated by T/- and A/- at positions 233 and 291 in the alignment. Because of this heterogeneity, the sequences could not be read between these positions for *Edw. ictaluri* isolates RE-33, AL-93-58 and EILO. However, the heterogeneity was slightly less for isolates IA-30-NJ#1 and ATCC-33202, making it possible to determine that the sequence between the heterogeneous positions is likely identical to that of 001-S99-1643. The two groups of sequences also differed from each other at position 211. Adenine predominated at position 211 in the sequences of the first group, whereas guanosine was present at similar or greater levels than adenine in sequences of the second group (indicated by R in the sequence). In addition to the ambiguous positions indicated in the figure, other positions were ambiguous in at least some of the *Edw. ictaluri* smaller ISR sequences; the predominant bases are shown for these other positions. The asterisk above position 105 indicates a single nucleotide difference between the $tRNA^{Glu}$ coding sequences in *Edw. ictaluri* and *Edw. tarda* ISR sequences and those of *Escherichia coli*, which contain C at this position. Box A indicates the consensus antiterminator box A sequence that is present in most bacterial 16S–23S ISR (Berg *et al.* 1989). Sequences were deposited in GenBank under the accession numbers indicated in Table 1

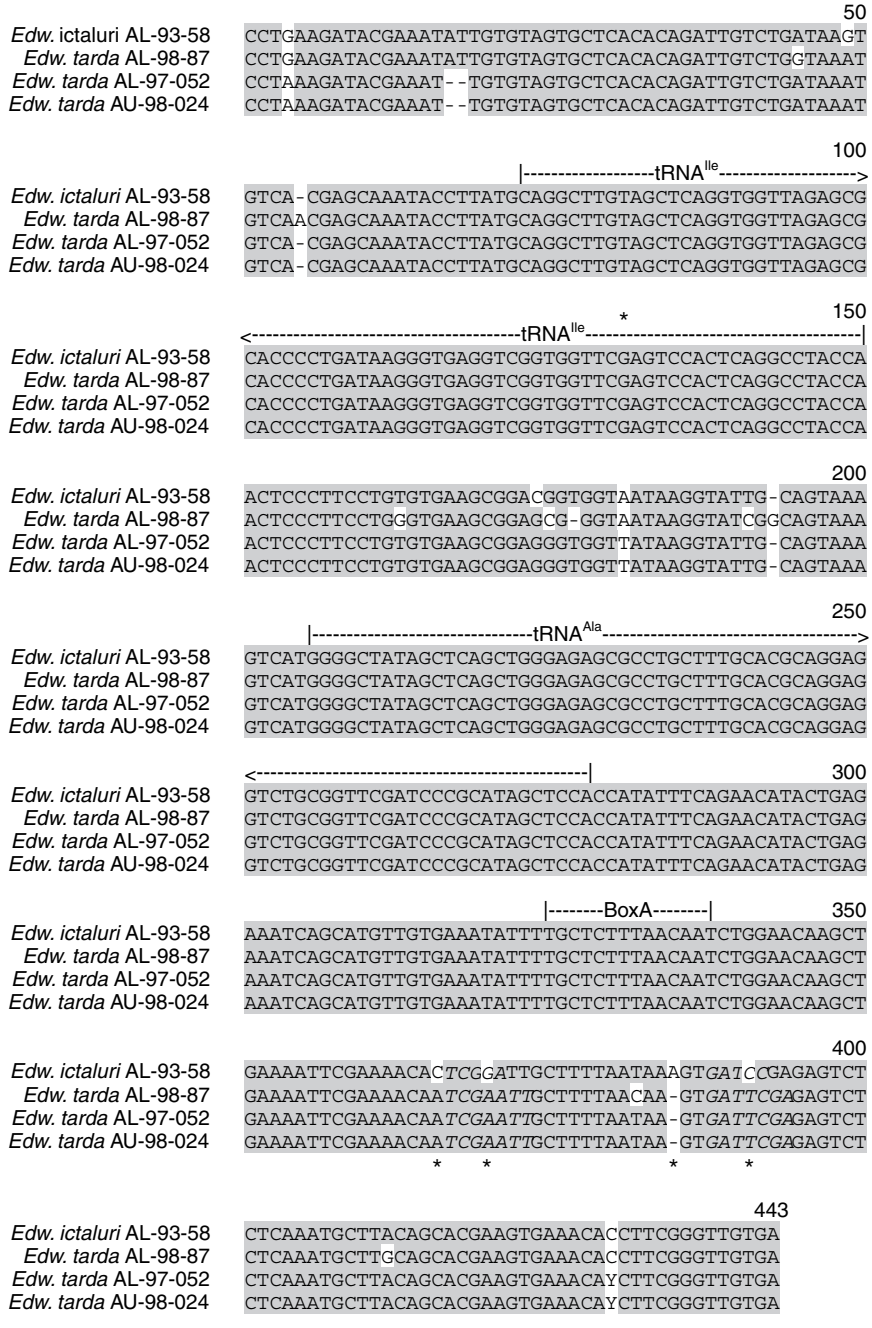

Fig. 3 Aligned sequences of *Edwardsiella ictaluri* and *Edwardsiella tarda* larger ISR. Dashes indicate gaps introduced to improve alignment. Shading indicates identical nucleotides in a majority of the sequences. Asterisks below the aligned sequences indicate positions where all *Edw. tarda* sequences determined were identical to each other but differed from those of *Edw. ictaluri*. Italics indicate restriction enzyme recognition sites present only in either *Edw. ictaluri* or *Edw. tarda* sequences, potentially useful for distinguishing between *Edw. ictaluri* and *Edw. tarda* (see Table 2). Some of the recognition sites are overlapping. The asterisk above position 131 indicates a single nucleotide difference between the $tRNA^{Ile}$ coding sequences in *Edw. ictaluri* and *Edw. tarda* ISR sequences and those of *Escherichia coli*, which contain A at this position. The $tRNA^{Ala}$ coding sequences of *Edw. ictaluri* and *Edw. tarda* ISR sequences are identical to those of *E. coli*. Box A indicates the consensus antiterminator box A sequence that is present in most bacterial 16S–23S ISR (Berg *et al.* 1989). Sequences were deposited in GenBank under the accession numbers indicated in Table 1

ictaluri sequences at these positions was not because of contamination with *Edw. tarda* sequences, because these sequences were not heterogeneous at other positions where the ISR of *Edw. ictaluri* and *Edw. tarda* differ. The sequences of the smaller ISR of the *Edw. tarda* isolates were 97% identical to the *Edw. ictaluri* sequences. Two of the three complete *Edw. tarda* 350-bp ISR sequences (AL-97-052 and AU-98-024) were identical to each other, except for one ambiguous position in the AU-98-024 sequence, and 97% identical to that of *Edw. tarda* AL-98-87. The partial sequence of *Edw. tarda* AL-03-032 350-bp ISR was also identical to those of AL-97-052 and AU-98-024 in the positions determined.

Comparison of large ISR sequences

The complete sequence of the larger ISR was determined for one *Edw. ictaluri* isolate, AL-93-58 (Fig. 3). Partial sequences of the 3′-portion of larger ISR from four other *Edw. ictaluri* isolates were identical to that of AL-93-58 in

Table 2 Restriction fragment length polymorphisms of PCR products potentially useful for distinguishing between *Edwardsiella ictaluri* and *Edwardsiella tarda*

Restriction enzyme	Recognition sequence	Position of polymorphism*	Fragment sizes (bp)		
			Edw. ictaluri	*Edw. tarda*	*Edw. tarda*†
*Hpy*188I	TCNGA	228	243 (L)	**418** (L)	**348** (S)
			233 (S)	**348** (S)	243 (L)
			174 (L, S)	**196** (L)	**196** (L)
			93 (L, S)	174 (S)	173 (L, S)
			81 (L)	75 (L, S)	75 (L, S)
			75 (L, S)		
			23 (L, S)		
*Tsp*509I	AATT	228	485 (L)	486 (L)	**336** (L)
			394 (S)	394 (S)	**262** (L, S)
			279 (L, S)	**262** (L, S)	**245** (S)
				16 (L, S)	**148** (L, S)
					16 (L, S)
*Taq*I	TCGA	228, 299	**275** (L, S)	263 (L)	
			262 (L)	**241** (L, S)	
			187 (S)	187 (S)	
			130 (L)	130 (L)	
			126 (S)	126 (S)	
			97 (L)	97 (L)	
			85 (S)	**64** (S)	
				23 (L, S)	
				21 (S)	
				10 (L, S)	
*Tfi*I	GAWTC	299	**668** (L)	**453** (L)	
			370 (S)	**244** (L, S)	
			207 (S)	207 (S)	
			67 (L, S)	**154** (S)	
			29 (L, S)	67 (L, S)	
*Sau*3AI	GATC	299	**307** (S)	**551** (S)	
			272 (L)	**370** (L)	
			245 (L, S)	273 (L)	
			126 (L)	121 (L, S)	
			121 (L, S)		
*Alw*I	$GGATC(N)_4$	299	**294** (S)	543 (S)	
			259 (L)	**375** (L)	
			250 (L, S)	260 (L)	
			129 (L, S)	129 (L, S)	
			126 (L)		

ISR, intergenic spacer region.
Bold letters indicate fragments unique to *Edw. ictaluri* or *Edw. tarda*.
L, generated from PCR product containing longer ISR; S, generated from PCR product containing shorter ISR.
*In alignment of shorter ISR shown in Fig. 2.
†Additional polymorphisms among *Edw. tarda* ISR sequences generate different restriction patterns for *Hpy*188I and *Tsp*509I. *Hpy*188Y pattern difference is because of a polymorphism at position 45 in the longer ISR (see Fig. 3). A two-nucleotide deletion at position 17 in the shorter and longer ISR of two *Edw. tarda* isolates generates an *Tsp*509I recognition site (see Figs 2 and 3).

the positions determined. The sequences of the *Edw. tarda* larger ISR were 96–98% identical to the *Edw. ictaluri* larger ISR. As was the case for the smaller ISR sequences, the larger ISR sequences of *Edw. tarda* AL-97-052 and AU-98-24 were identical to each other and differed 3% from that of *Edw. tarda* AU-98-87 (the sequence of the larger ISR was not determined for the fourth *Edw. tarda* isolate).

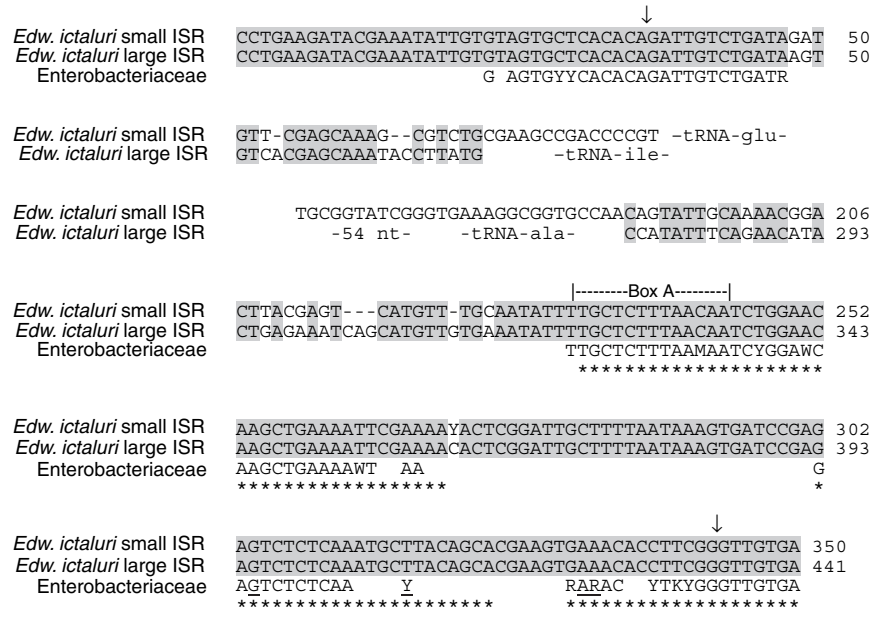

Fig. 4 Alignment of sequences of *Edwardsiella ictaluri* smaller and larger ISR sequences flanking the tRNA-coding sequences. Dashes indicate gaps introduced to improve alignment. Shading indicates identical nucleotides in the smaller and larger ISR. Enterobacteriaceae indicates the consensus for positions conserved among ISR sequences of a wide variety of Enterobacteriaceae (listed in Table 3). In the consensus, underlining indicates that 18 of the 19 Enterobacteriaceae ISR sequences compared with *Edw. ictaluri* agree with the consensus (nonunderlined nucleotides indicate that all 19 of the other Enterobacteriaceae ISR sequences agree with the consensus). R = A or G; Y = C or T; M = A or C; K = G or T; W = A or T. Box A indicates the consensus antiterminator box A sequence that is present in most bacterial 16S–23S ISR (Berg *et al.* 1989). Arrows indicate putative RNase III cleavage sites; *, represents the conserved regions used for phylogenetic analyses

Restriction fragment length polymorphisms

Analysis of restriction endonuclease recognition sites revealed that the few nucleotide differences between *Edw. ictaluri* and *Edw. tarda* ISR resulted in differential presence of recognition sites for several commercially-available restriction enzymes, which might be used for differentiation of the two species. The resulting restriction fragment length polymorphism (RFLP) are shown in Table 2. Differences among isolates of *Edw. tarda* resulted in more than one restriction fragment pattern for *Edw. tarda* ISR PCR products for two of the restriction enzymes, but each pattern is distinct from the pattern for *Edw. ictaluri* ISR PCR products.

Comparison of *Edw. ictaluri* ISR sequences to those of other Enterobacteriaceae

Sequences of the smaller and larger ISR of *Edw. ictaluri* were identical to each other for the 47 positions immediately following 16S rRNA coding sequences and the last 126 positions immediately preceding the 23S rRNA coding sequences, beginning seven nucleotides before box A (Fig. 4). Similarity of the two ISR was evident through the first 62 nt of the larger ISR, almost to the beginning of the first tRNA, and similarity resumed after the second tRNA of the larger ISR. Within these regions of identity between the larger and smaller ISR, blocks of sequence conserved among a wide variety of Enterobacteriaceae members (listed in Table 3) are found (see Fig. 4). These regions include the consensus antiterminator box A that is present in most bacterial 16S–23S ISR (Berg *et al.* 1989) and those predicted to participate in secondary structure with regions before the 16S rRNA coding region and after the 23S rRNA coding region, which also include RNase III cleavage sites (Brosius *et al.* 1981). The regions conserved between *Edw. ictaluri* and other Enterobacteriaceae following box A are interrupted by stem and loop structures in secondary structure models for ISR of *Eschericia coli* and other Enterobacteriaceae (Brosius *et al.* 1981; Luz *et al.* 1998). The corresponding sequences of the *Edw. ictaluri* ISR also have the potential to form similar stem and loop structures (not shown).

The sequences of the $tRNA^{Glu}$ genes found in the smaller ISR of *Edw. ictaluri* and *Edw. tarda* were identical to the $tRNA^{Glu}$ gene found in the ISR of several other Enterobacteriaceae (*Photorhabus luminescens*, *Proteus mirabilis* and *Providencia stuartii)* as well as several *Aeromonas* species. They differed from the $tRNA^{Glu}$ sequence found in ISR of *E. coli* rrn B, C, E and G at one position, indicated in Fig. 2. The sequences of the $tRNA^{Ala}$ genes found in the larger ISR of *Edw. ictaluri* and *Edw. tarda* were identical to those found

Table 3 Enterobacteriaceae 16S rRNA and ISR sequences used for comparison with *Edwardsiella ictaluri*

Species	GenBank accession no.		Number of tRNAs in ISR*
	16S rRNA	ISR	
Arsenophonus endosymbiont of *Tetraleurodes acaciae*	AY264670	AY264670	2
Citrobacter freundii	AJ233408	AF047423	0
Enterobacter aerogenes	AJ251468	AF047426	0
Erwinia amylovora	AJ010485	AJ010485	1
Escherichia coli K12	U00096	U00096	2
Klebsiella pneumoniae	X87276	AF047425	1
Pantoea stewartii	AF373198	AJ311838	1
Pectobacterium atrosepticum	AF373181	AF373166	2
Pectobacterium cacticidum	Z96092	AF234275	2
Pectobacterium chrysanthemi	AF373203	AF373203	1
Pectobacterium cypripedii	Z96094	AF234276	2
Photorhabdus luminescens laumondii	BX571873	BX571873	2
Proteus vulgaris	X07652	AY116926	2
Providencia stuartii	AF008581	AY116923	1
Salmonella serovar Typhi Ty2	AE016842	AE016842	2
Salmonella enterica strain LT2	AF046814	AF046814	2
Shigella flexneri	AE015280	AE015280	2
Yersinia enterolytica	M59292	AY116924	1
Yersinia pestis	AE013694	AE013694	2
Hafnia alvei	AY572428		
Cedecea davisae	AF493976		
Cedecea neteri	AB086230		
Enterobacter cancerogenus	Z96078		
Enterobacter sakazakii	AB004746		
Klebsiella terrigena	Y17658		
Klebsiella trevisanii	X93216		
Leclercia adecarboxylata	AJ277978		
Serratia marescens	AJ550467		
Serratia plymuthica	AJ233433		
Serratia rubidaea	AJ233436		
Trabulsiella guamensis	AY373830		

ISR, intergenic spacer region.
*ISR containing one tRNA coding sequence contain the coding sequence for $tRNA^{Glu}$; those containing two tRNA coding sequences contain those for $tRNA^{Ile}$ and $tRNA^{Ala}$. ISR sequences containing two tRNAs were used whenever available.

in *E. coli* rrn A, D and H. However, the $tRNA^{Ile}$ genes of *Edw. ictaluri* and *Edw. tarda* differed from those of *E. coli* at one position, indicated in Fig. 3, and were not identical to sequences of any bacterial tRNase coding sequences found in GenBank.

The BLASTn searches of GenBank using *Edw. ictaluri* sequences of the entire 16S rRNA gene (obtained from GenBank, accession no. AF310622), the entire longer ISR, or the portion of the longer ISR following the second tRNA consistently yielded the highest scores for sequences from members of the genus *Pectobacterium*. Other species that received high scores in BLASTn searches using *Edw. ictaluri* or *Edw. tarda* ISR sequences (but not 16S rRNA sequences) were primary endosymbionts of *Sitophilus zeamais* or *Sitophilus oryzae* and *Photorhabdus luminescens*. However, phylogenetic analyses by neighbour-joining and maximum-likelihood methods failed to support the contention that *Edwardsiella* species are more closely related to any of these species than to other members of the family Enterobacteriaceae (results not shown).

DISCUSSION

Conventional methods for determining differences within the species *Edw. ictaluri* and *Edw. tarda* based on phenotypic characteristics have produced equivocal results and very few studies have attempted to distinguish between the two species. The relative divergence in size and sequence of the

ISR among different groups of procaryotes, together with its position between conserved rRNA genes makes it ideal to detect subtle differences within and between species at the molecular level (Barry *et al.* 1991; Gutler and Stanisich 1996). In the present study, the 16S–23S rRNA ISR of 19 *Edw. ictaluri* and four *Edw. tarda* isolates from fish in four geographic regions were examined for interspecies and intraspecific differences. Generally, ISR length polymorphisms are less conspicuous among strains of a given species than between different species of the same genus (Gutler and Stanisich 1996; Scheinert *et al.* 1996; Naimi *et al.* 1997; Daffonchio *et al.* 1998). However, we detected length polymorphisms neither within species nor between these two species of the genus *Edwardsiella*. Sequence analysis revealed that the *c.* 700-bp products consisted of ISR of 350 bp, which contained a $tRNA^{Glu}$ gene, while the less abundant *c.* 800-bp products included ISR of 441 bp, which contained $tRNA^{Ile}$ and $tRNA^{Ala}$ genes. The tRNA genes in the respective ISR are arranged similarly to the tRNA genes found in ISR1 and ISR2 in *E. coli* (Anton *et al.* 1998). Identification of two kinds of ISR indicates that *Edwardsiella* species have at least two *rrn* operons. However, the number of *rrn* operons was not determined, and each PCR product was likely derived from multiple *rrn* operons. The difference in relative abundance of the PCR products from the two types of ISR might reflect a higher number of ISR1-containing *rrn* operons relative to ISR2-containing *rrn* operons in *Edwardsiella* species. Alternatively, the difference might be a result of different amplification efficiencies of the two types of ISR under our PCR conditions.

As expected from the similarity in size of the PCR products containing either the longer or shorter ISR among the *Edw. ictaluri* and *Edw. tarda* isolates, the sequences do not contain large insertions/deletions relative to each other that are notably present in some other Enterobacteriaceae species such as *E. coli* (Anton *et al.* 1998), and *Salmonella enterica* (Luz *et al.* 1998). In the latter two genera, insertions/deletions of blocks of nucleotides in the *rrn* operon have been observed, generally located at equivalent regions of the putative secondary structure (Luz *et al.* 1998). Instead, differences among *Edw. tarda* ISR and between *Edw. ictaluri* and *Edw. tarda* ISR are confined to point mutations and single nucleotide insertions/deletions. The sequences of the smaller ISR from the different *Edw. ictaluri* isolates are essentially identical to each other, even though two of the isolates (EILO and IA-30-NJ#1) were isolated from different fish species (*Clarias batracus* and *Noturus gyrinus* respectively) on a different continent or different geographical region of the US than the others. We also did not obtain any evidence of sequence heterogeneity in the larger ISR among *Edw. ictaluri* isolates. The only differences in the shorter ISR sequences among *Edw. ictaluri* isolates are in the degree of heterogeneity at individual positions in the sequences. Five of the *Edw. ictaluri* isolates (IA-30-NJ#1, RE-33, AL-93-58, EILO and ATCC-33202) each exhibit marked heterogeneity in the number of Ts (three or four) at positions 230–233 and number of As (two or three) at positions 291–293 in the alignment. It should be noted that ATCC-33202 represents the type strain of *Edw. ictaluri* (Hawke *et al.* 1981), which has undergone numerous *in vitro* passages, and RE-33 is a vaccine strain derived following 30 passages *in vitro* from the parent strain EILO, isolated from a walking catfish (*C. batracus*) in Thailand (Klesius and Shoemaker 1999). It is likely that the remaining three isolates of *Edw. ictaluri* exhibiting heterogeneity in number of Ts and As at these positions have been similarly passaged, as they remained in the laboratory for a long time. It is possible that the heterogeneity observed developed during *in vitro* passage. Because DNA was prepared from cultures inoculated from single colonies, it is unlikely the heterogeneity represents heterogeneity among the bacteria, and more likely that it reflects heterogeneity among *rrn* operons. The other 14 *Edw. ictaluri* isolates have predominately four Ts and three As at the positions denoted above. All but one *Edw. tarda* isolate (AU-98-87), have three Ts and two As at the corresponding positions. For both the shorter and longer ISR sequences, two of the *Edw. tarda* isolates (AL-97-052 and AU-98-024) are identical to each other, except for a single ambiguous position in the AU-98-024 shorter ISR sequence, but differ 3% from those of *Edw. tarda* AU-98-87. The presence of some variability among ISR sequences of *Edw. tarda* isolates is in contrast to the identity of ISR sequences among *Edw. ictaluri* isolates and is consistent with the phenotypic and genotypic heterogeneity of *Edw. tarda* reflected by serotyping and random amplified polymorphic DNA profiles (Sakazaki 1984; Tamura *et al.* 1988; Nucci *et al.* 2002). Results of our recent phenotypic characterization of the same isolates examined here, as well as additional *Edw. tarda* isolates, by comparison of total protein SDS-PAGE profiles and of immunoblot profiles, also indicate relative homogeneity of *Edw. ictaluri* isolates in contrast to heterogeneity of *Edw. tarda* isolates (Panangala *et al.* 2005).

Although we expected to find great similarity among *Edw. ictaluri* ISR sequences, we were surprised to find 100% identity, because previous phenotypic and genotypic characterization of some of the *Edw. ictaluri* isolates examined here had revealed differences. Specifically, isolate AL-93-58 was able to cause mortality in catfish vaccinated with live EILO, whereas several other isolates (including numbers 2, 13, 15, and 17 in Table 1) were not, suggesting virulence or antigenic differences between AL-93-58 and the other isolates tested (Klesius and Shoemaker 1999). AFLP analysis showed that isolates from tadpole madtom, including IA-30-NJ#1, differs (<80% similarity) from isolates from catfish in Mississippi (including numbers 3–7 in

Table 1) and EILO (Klesius *et al.* 2003). However, these phenotypic and genotypic differences are not reflected in ISR sequences, which are identical to other *Edw. ictaluri* isolates.

Our small sample of four *Edw. tarda* isolates revealed two groups based on ISR sequences. Our recent phenotypic characterization of three of these and additional *Edw. tarda* isolates also revealed two groups (Panangala *et al.* 2005). However, the groups generated by the different phenotypic and genotypic analyses do not coincide.

The sequences of the smaller ISR of *Edw. tarda* and *Edw. ictaluri* are 97% identical, and those of the longer ISR 96–97% identical. It should be noted that for both the smaller and larger ISR sequences, the degree of difference among *Edw. tarda* isolates is similar to the degree of difference between *Edw. ictaluri* and *Edw. tarda*. The high degree of sequence similarity in the ISR between *Edw. ictaluri* and *Edw. tarda* likely reflects descent from a phylogenetically closely related common ancestor in the Enterobacteriaceae lineage. However, our phylogenetic analyses of available ISR sequences did not identify members of other genera closely related to the putative common ancestor. Abbott and Janda (2001) approached the question of the phylogeny of the genus *Edwardsiella* by conducting a BLAST search of the 16S rRNA sequences available in the MicroSeq database and conducting a phylogenetic analysis, based on the neighbour-joining method, of the twenty sequences yielding the highest scores. They concluded, based on this analysis, that the most closely related species to *Edwardsiella* were *Trabulsiella guamensis* and *Enterobacter sakazakii*. However, no bootstrap values were provided. Unfortunately, ISR sequences are not available for any of the 20 species identified by Abbott and Janda's BLAST search. However, our BLASTn search of GenBank using the entire 16S rRNA gene sequence of *Edw. ictaluri* generated the highest scores for different species than those Abbott and Janda identified, and many of them had ISR sequences available. Our phylogenetic analyses, by neighbour-joining and maximum-likelihood methods, of the 16S rRNA sequences of some of the species identified by Abbott and Janda (species numbers 21–31 in Table 2), those identified by our BLASTn searches as being most similar to *Edw. ictaluri*, and other representative members of Enterobacteriaceae, failed to generate support for a close relationship between *Edwardsiella* species and any particular members of Enterobacteriaceae. Likewise, our phylogenetic analysis of three relatively conserved regions of ISR between the second tRNA and 23S rRNA coding sequences failed to reveal close relatives of *Edwardsiella*.

The identity of ISR sequences among epidemiologically unrelated *Edw. ictaluri* isolates precludes use of RFLP analysis of ISR PCR products to compare isolates of *Edw. ictaluri*. However, we identified restriction endonuclease recognition sites that differ between ISR of *Edw. ictaluri* and *Edw. tarda*, making it possible to distinguish between these closely-related species by RFLP analysis of ISR PCR products. To our knowledge, this is the first study to comparatively analyse the ISR of *Edw. ictaluri* and *Edw. tarda*.

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